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Experiences With Pro-Urokinase and Potentiation of Its Fibrinolytic Effect by Urokinase and by Tissue Plasminogen Activator

VICTOR GUREWICH, MD

Boston, Massachusetts

Pro-urokinase is a single chain, precursor form of two chain, 54,000 M_r urokinase. Although originally isolated from urine, pro-urokinase is also found in blood, where it is believed to participate in natural fibrinolysis alongside tissue plasminogen activator (t-PA). These two plasminogen activators share the property of inducing fibrin-selective plasminogen activation, but many of their other properties, including their modes of action, are dissimilar. A comparison of some of the clinically relevant properties of pro-urokinase and t-PA is provided.

A multicenter, dose-finding clinical trial of native pro-urokinase is underway in the United States and in West Germany. At the time of this writing, 110 patients with angiographically proved acute coronary thrombosis have been treated. The findings from one center are summarized in some detail and the overall experience is reviewed. Preliminary evidence for a potentiating effect on pro-urokinase–induced thrombolysis by urokinase is presented. The findings suggest that a bolus of urokinase (200,000 IU) at the outset increases the reperfusion rate from 60 to >80% and shortens the lysis time from about 50 to 30 minutes. A modest (19%) but significant ($p < 0.05$) decrease in fibrinogen accompanied thrombolysis by urokinase/pro-urokinase. Nonetheless, significant bleeding complications in the multicenter study have, to date, not been encountered and it is suggested that this may be related to pro-urokinase's sensitivity to inactivation by thrombin and lack of potentiation by heparin.

Some in vitro data are summarized that provide an explanation for the potentiating effect of urokinase, the mechanism for which is believed to be similar to that by which synergism between t-PA and pro-urokinase has been postulated to occur. Evidence is reviewed that indicates that pro-urokinase and t-PA induce thrombolysis far more efficiently when they are combined than when each is used alone. The findings suggest that a bolus of t-PA, in place of urokinase, followed by an infusion of pro-urokinase may be optimal because t-PA is more effective in triggering lysis and pro-urokinase is more reactive after fibrinolysis has started.

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Currently available plasminogen activators, streptokinase and urokinase, are effective thrombolytic agents that have been on the market in this country for 10 years but have not enjoyed much popularity. Each activator induces hemostatic abnormalities that are related to the systemic activation of plasminogen. The resultant hemorrhagic diathesis has been sufficiently troublesome to clinicians so as to seriously limit the use of these agents. Concern for safety, rather than efficacy, has therefore been the principal obstacle to thrombolytic therapy.

By contrast to the effects of streptokinase and urokinase, spontaneous fibrinolysis, which can be stimulated by vigorous exercise, cuff occlusion or other measures that induce release of tissue plasminogen activator, is highly fibrin specific and efficient (1–3). Therefore, nature's design for inducing clot lysis serves as a good model to be emulated by therapeutic thrombolysis. The two natural activators of fibrinolysis that have been isolated from blood are tissue plasminogen activator (t-PA) (4) and pro-urokinase (5). Thanks to modern cell culture methods and recombinant DNA technology, both of these activators have been made available in sufficient quantities to allow for their clinical evaluation, and much information on recombinant t-PA (rt-PA) has already been published. Experience with pro-urokinase is more limited, and it is not known which of these two natural activators at pharmacologic doses is most effective, efficient and specific. Second, the use of combinations of rt-PA and pro-urokinase, which replicate nature's design, are only starting to be considered.

From the Vascular Laboratory, Department of Biomedical Research, St. Elizabeth's Hospital, Tufts University School of Medicine, Boston, Massachusetts. This study was supported by Grant HL23367-08 from the National Institutes of Health, Bethesda, Maryland.

Address for reprints: Victor Gurewich, MD, Vascular Laboratory, St. Elizabeth's Hospital, 736 Cambridge Street, Boston, Massachusetts 02135.

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Pro-Urokinase

Fibrinolytic Properties

Pro-urokinase is a single chain precursor of classical, two chain 55,000 M_r urokinase. Although the latter has been known since 1951 (6), pro-urokinase eluded detection until 1979 when it was first isolated and characterized (7,8). Pro-urokinase has very different fibrinolytic properties from those of urokinase in that it induces fibrin-specific clot lysis (9–11). Fibrin selectivity comparable with that found in animals has recently been reported from pilot studies in humans (12,13). Although pro-urokinase and t-PA are each fibrin-selective activators of plasminogen, in most other respects they are quite dissimilar and they induce fibrinolysis by different mechanisms. A summary of certain properties that are of potential clinical interest are provided in Table 1.

Antigenicity. Because pro-urokinase is an endogenous protein like urokinase and t-PA, it has not been found to be antigenic even at pharmacologic doses, in contrast to streptokinase. The many years of clinical experience with urokinase provide additional reassurance that antigenicity is unlikely to be encountered. In contrast to t-PA, pro-urokinase is a zymogen and is stable in plasma (14). Specifically, it does not form sodium dodecylsulfate-stable complexes with inhibitors and its half-time to inhibition is >72 hours (9). However, despite its inert nature in blood, pro-urokinase is rapidly cleared from the circulation and has a clearance half-time that is similar to that of t-PA. This means that the fibrinolytic effects of pro-urokinase will dissipate promptly (within 15 minutes) after stopping an infusion.

Fibrin specificity. Although pro-urokinase was first isolated because it had an affinity to fibrin/Celite greater than that of urokinase (7,8), pro-urokinase does not have a fibrin clot affinity comparable with that of t-PA, and the fibrin specificity of pro-urokinase does not depend on fibrin binding. Rather, it appears to be related to a selective activation of fibrin-bound plasminogen because of the latter's conformational change (14). These contrasting mechanisms of action form the basis for their synergy (see later). Unlike t-PA, pro-urokinase–induced clot lysis in vitro is associated with a lag phase followed by a rapid second phase of lysis. This lag phase has been attributed to the fact that virgin fibrin has little plasminogen bound to it, providing little substrate for pro-urokinase (15). After degradation has been initiated, many new plasminogen binding sites are exposed on fibrin (16) allowing pro-urokinase to become increasingly more effective.

Table 1. Pro-Urokinase and Tissue-Type Plasminogen Activator Comparison of Some Properties of Potential Clinical Relevance

	Pro-UK	t-PA
Relative fibrin specificity	+	+
Nonantigenicity	+	+
Short in vivo half-time	+	+
Fibrin binding	+/−	+
Stability in plasma	+	−
Immediacy of onset	−	+
Inactivation by thrombin	+	−
Potentiation of nonspecificity by heparin	−	+

Pro-UK = pro-urokinase; t-PA = tissue-type plasminogen activator.

Inactivation by thrombin: lower tendency to bleeding. Finally, pro-urokinase is very sensitive to inactivation by thrombin (17) so that at a site of active hemostasis, where thrombin is being generated, pro-urokinase will be promptly neutralized. This may explain the seemingly lower tendency to induce bleeding encountered in the preliminary clinical investigations with pro-urokinase as compared with that reported for rt-PA–induced thrombolysis (18). Moreover, nonspecific plasminogen activation by pro-urokinase is not potentiated by heparin (unpublished observations), whereas heparin has been shown to bind both rt-PA and plasminogen and thereby facilitate nonspecific plasminogen activation by t-PA (19). Heparin and pro-urokinase have, in fact, been infused simultaneously without complications (see later).

Clinical Studies of Pro-Urokinase

Preliminary studies. In six patients with acute coronary thrombosis (12), 40 mg of pro-urokinase was infused over 60 minutes, followed by an additional 20 mg over 30 minutes in three of the patients. The infusion was associated with reperfusion in four patients; significant fibrinogen degradation occurred in one. The native pro-urokinase used in the study contained about 5% urokinase, which may have contributed to the fibrinogen degradation. In a second study by Van de Werf et al. (13), 17 patients with acute Q wave myocardial infarction were treated with nonglycosylated, recombinant pro-urokinase expressed in *Escherichia coli*. A total of 40 mg was infused in eight patients and it induced incomplete coronary recanalization in six of them. In an additional nine patients, infusion of 70 mg induced recanalization with normal distal filling in seven. This was, however, accompanied by 50% reduction in fibrinogen. It has not yet been determined whether the fibrinolytic specificity of recombinant pro-urokinase is comparable with that of native pro-urokinase.

Multicenter study. At the time of this writing, 110 patients with acute coronary thrombosis and infarction have been treated with native pro-urokinase in the multicenter dose-finding pilot studies being conducted by Sandoz Pharmaceuticals in the United States and in West Germany. The material used in these studies was purified from the culture medium of a human kidney tumor cell line and has a molecular weight of approximately 54,000. It migrates as a single band on sodium dodecylsulfate-polyacrylamide gel under reducing conditions and has an amidolytic activity <0.5% of its activated form, indicating that it contained negligible urokinase contaminant. The manuscript (submit-

JACC Vol. 10, No. 5
November 1987:16B–21B

Table 2. Pro-Urokinase (60 minute infusion) in 20 Cases of Acute Coronary Thrombosis*

Patient	Dose (mg)	No. With Reperfusion	Time (min) to Reperfusion
Group I			
4	15	2	50, 60
1	22	—	—
3	30	2	60
1	60	1	—
9		55%	57
Group IV†			
11	48.4	9	13 to 60
		82%	30

*Unpublished observations of Kaspen et al. Cited with permission.
†Bolus urokinase (200,000 IU) at onset. All patients received heparin (1,250 units/h) for 24 hours. Reocclusion at 24 hours: 0 of 18.

ted for publication) from one of the participating centers, by Kaspar et al. of Freiburg, West Germany, has been selected for summary with the kind permission of the principal investigator, because it illustrates some findings of special interest.

In the first phase of this study (Table 2), a total dose ranging from 15 to 60 mg was infused over 1 hour in nine patients (Group I). This resulted in grade 3 (TIMI classification) coronary reperfusion in three patients and grade 2 reperfusion in an additional two patients, giving an overall reperfusion rate of 55%. A relatively long time to reperfusion of 57 minutes was found. No fibrinogen degradation occurred in this group. These overall findings are representative of the experience from the other centers. A high degree of specificity and no bleeding, but a relatively low reperfusion rate and long time to reperfusion, has been found when 30 to 50 mg of pro-urokinase was infused over 1 hour. At doses of 70 mg/h, significant fibrinogen degradation has been seen in some patients.

To improve the success rate and shorten the lysis time, it was proposed by Dr. Welzel of Sandoz Nurnberg that a bolus dose of urokinase (200,000 IU) be administered at the outset. At this dose, urokinase has no demonstrable independent effect. Among the subsequent 11 patients (Group II) who received an infusion of 48.4 mg of pro-urokinase over 1 hour after the urokinase bolus, grade 3 reperfusion occurred in 9 patients and a mean time to clot lysis of 30 minutes was observed. Although Groups I and II (Table 2) cannot be considered comparable in that Group II received a higher dose of pro-urokinase, the findings from the other centers indicate that 50 mg of pro-urokinase is associated with reperfusion in about 60% of patients and 70 mg in about 65%, with a mean time to lysis of about 45 minutes. Taken together, these results suggest that the high lysis rate and shorter lysis time found in Group II are attributable more to the augmenting effect of urokinase on pro-urokinase–induced thrombolysis than to differences in pro-urokinase dosage. Moreover, subsequent to submission of this manuscript, seven more patients have been treated to date with this urokinase/pro-urokinase combination by Kasper et al., bringing the total to 18 patients, of whom 15 (83%) had reperfusion at about 30 minutes.

The combination urokinase/pro-urokinase used in phase II was accompanied by a 19% decrease in fibrinogen concentration at 60 minutes ($p < 0.05$) compared with baseline. Of additional interest is that all patients in the Freiburg study received simultaneous heparin (1,250 U/h), which was continued for 24 hours (Table 2). Bleeding around a catheter was reported in one of the patients. No other bleeding complications were encountered. Repeat coronary angiography was done at 24 to 48 hours in 18 of the patients, and no evidence of reocclusion was found. This experience suggests that the combination of high dose heparin with pro-urokinase may offer a solution to the vexing problem of rethrombosis.

Mechanism for the Synergistic Effect of Urokinase or of t-PA on Pro-Urokinase

The clinical findings from phase II of the multicenter study summarized are supported by in vitro observations that provide an explanation for the potentiating effect of urokinase on pro-urokinase–induced thrombolysis. The mechanism involved is believed to be similar to that previously proposed to account for the synergism between

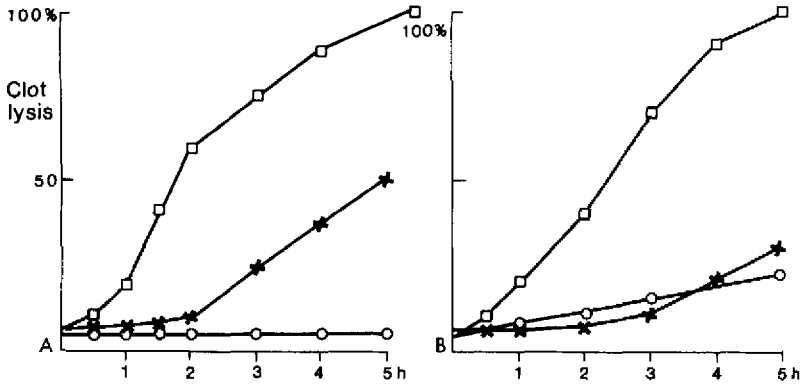


Figure 1. A, Lysis of radiolabeled fibrin clots by 20 IU/ml urokinase (○), 80 IU/ml pro-urokinase (**X**) or by a combination of urokinase and pro-urokinase (□). **B,** Clot lysis by 3 U/ml t-PA (○), 40 IU/ml pro-urokinase (**X**) or by a combination of t-PA and pro-urokinase (□). A substantially greater than an additive effect is apparent with both combinations. These combinations also fulfilled the mathematical definition of synergy (sum of the fractional combinations were <1, actually about 0.7) (20).

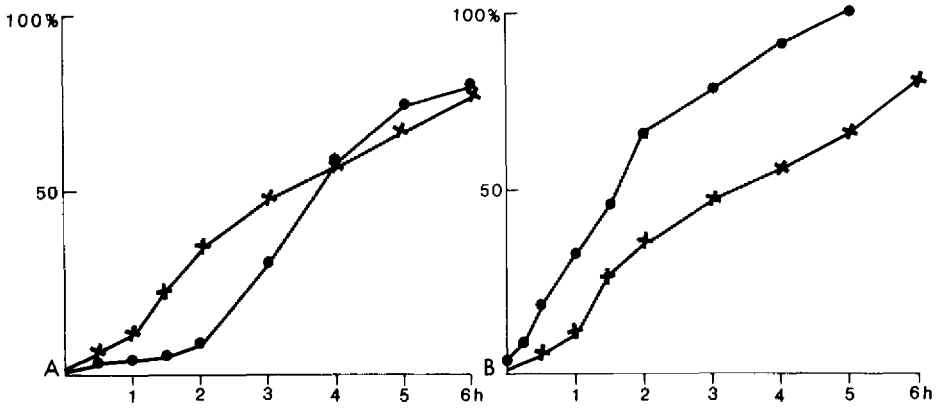


Figure 2. A, Lysis of radiolabeled fibrin clots by 300 ng/ml pro-urokinase (●) or by 50 ng/ml t-PA (**X**). The longer lag phase and steeper second phase of lysis by pro-urokinase is evident. **B,** Lysis of clots preincubated with t-PA (50 ng/ml) to promote binding. These clots were washed and then incubated in plasma containing 50 ng/ml t-PA (**X**) or 300 ng pro-urokinase (●). Marked potentiation of clot lysis by pro-urokinase but not by the additional t-PA is shown.

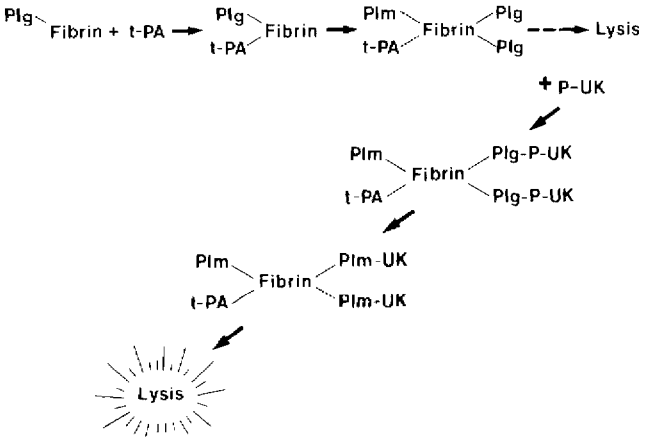
t-PA and pro-urokinase (15). A summary of these experiments is included here because they are of potential clinical importance and help interpret the observed promotion of pro-urokinase efficacy by urokinase.

Comparison of synergistic effect of urokinase or t-PA in pro-urokinase. The lytic effect of mixtures of urokinase and pro-urokinase or of t-PA and pro-urokinase were compared with the equivalent lytic effect of each activator alone in a previously standardized clot lysis assay system (9) (Fig. 1). The potentiation of clot lysis by either urokinase or t-PA on pro-urokinase obeyed Berenbaum's mathematical definition of synergy (20). That is, the same thrombolytic effect could be obtained by urokinase plus pro-urokinase or by t-PA plus pro-urokinase at algebraic fractional combinations <1 (Fig. 1). Moreover, the synergistic effect of mixtures of urokinase or t-PA with pro-urokinase could be reproduced when they were used sequentially rather than simultaneously. Pretreatment of a clot by urokinase, or plasmin followed by incubation of the washed clot with pro-urokinase in fresh plasma, resulted in clot lysis comparable with that obtained with simultaneous combinations of the activators. This effect was attributed to the exposure of new plasminogen binding sites on fibrin by plasmin generated by pretreatment with urokinase.

Differences between the action of pro-urokinase and rt-PA. By contrast, pretreatment of a fibrin clot with plasmin did not potentiate clot lysis by t-PA, suggesting that the plasminogen bound to the plasmin-exposed binding sites on fibrin is susceptible to activation by pro-urokinase but not by t-PA. This potentially important difference between pro-urokinase and t-PA is further illustrated by the results of another experiment (Fig. 2) that was designed to mimic the effect of a bolus of t-PA followed by an infusion of pro-urokinase. For this, clots were first exposed to t-PA (50 ng/ml of plasma) for 1 hour to provide time for binding of rt-PA to fibrin. After washing, the clot was incubated in fresh plasma with no additions, or with additional t-PA (50 ng/ml) or pro-urokinase (300 ng/ml). The additional rt-PA did not potentiate lysis over that induced by a single exposure to t-PA at this same concentration. The added t-PA evidently failed to augment the binding site occupancy of t-PA already present on the preincubated clot. By contrast, pro-urokinase induced marked potentiation of clot lysis relative to that induced by t-PA or pro-urokinase alone (Fig. 2b). The lytic effects of 50 ng t-PA and 300 ng of pro-urokinase in this in vitro system were equivalent, although t-PA clot lysis is relatively linear whereas that induced by pro-urokinase is sigmoidal (Fig. 2a). The difference in the dose requirements of t-PA and pro-urokinase to achieve comparable clot lysis in vitro have not been found in vivo.

Other investigators have failed to find synergism between t-PA and pro-urokinase in vitro (21) but have reported its

Figure 3. A virgin clot, represented at the **upper left,** contains little fibrin-bound plasminogen. However, its lysis is effectively initiated by t-PA, which appears to bind to a site close to the available plasminogen (Plg). The resultant plasmin (Plm) induces fibrin degradation, thereby exposing new plasminogen binding sites. The plasminogen bound to these sites is activatable by pro-urokinase but appears not to be accessible to t-PA, whereas the plasminogen on the virgin clot is resistant to activation by pro-urokinase. Clot lysis by the pro-urokinase–mediated pathway is additionally accelerated by pro-urokinase activation to urokinase, on the clot surface as shown. It is postulated that these complementary modes of action of t-PA and pro-urokinase illustrated explain their synergism.



JACC Vol. 10, No. 5
November 1987:16B–21B

occurrence in vivo (22,23). The discrepancy in the in vitro findings can be explained by differences in those experimental conditions that are essential for the demonstration of synergy. These include a high molar ratio of pro-urokinase to t-PA (≥5:1) and a pro-urokinase preparation free of urokinase contamination, because the latter is itself synergistic with pro-urokinase (24).

The apparent synergy, the contrasting shapes of the clot lysis curves of t-PA (linear) and pro-urokinase (sigmoidal) and the sharp differences in their fibrinolytic response to pretreatment of fibrin with plasmin, urokinase or t-PA are each expressions of dissimilar modes of action for t-PA and pro-urokinase. More specifically, we have postulated from these and other experimental findings that t-PA and pro-urokinase have different plasminogen substrates on the fibrin clot (25). A schema illustrating this hypothesis according to which t-PA and pro-urokinase are complementary in their fibrinolytic effect is shown in Figure 3.

An undegraded, virgin clot has little plasminogen bound to it, probably because no terminal lysine residues are present. The location of the plasminogen available is such that it is accessible to activation by fibrin-bound t-PA, presumably because the latter is bound to a neighboring region. By contrast, experimental evidence indicates that this plasminogen is resistant to activation by pro-urokinase. The plasmin generated by t-PA exposes new plasminogen-binding sites on fibrin, as previously shown by Harpel et al. (16). Once these new sites are occupied by plasminogen, a conformational change is induced in the Glu-plasminogen molecule, and it becomes susceptible to selective activation by pro-urokinase (14,26). On the other hand, these new plasminogen molecules appear to be inaccessible to activation by the fibrin-bound t-PA. Therefore, although clot lysis by t-PA alone will go on to completion as illustrated, it will be relatively inefficient and lysis will proceed much faster if pro-urokinase is added. The latter binds to the newly available plasminogen and activates it. In the process, pro-urokinase is converted to its more active derivative, urokinase, as shown, which further accelerates local plasmin generation and clot lysis. In short, t-PA is the trigger that efficiently initiates lysis and prepares the fibrin for pro-urokinase, which more effectively completes the lytic process (Fig. 3).

Clinical implications. The proposed model for the complementary and synergistic effects of t-PA and pro-urokinase explains a number of clinical findings such as the high dose requirements for t-PA and pro-urokinase when they are used alone for thrombolysis. Blood concentrations that are about 1,000-fold higher than their physiologic concentrations are being used in clinical trials. An explanation for this apparent inefficiency is provided by the schema. The model also explains the potentiation of pro-urokinase–induced thrombolysis by urokinase. The effect of urokinase mimics that of t-PA in that it generates the same new plasminogen-binding sites that are required for pro-urokinase. However, because urokinase does not bind to fibrin and is rapidly inactivated by inhibitors, its effect may be anticipated to be less efficient and less specific than that of t-PA. The potentiating effect of urokinase on pro-urokinase also implies, of course, that the urokinase contaminant in a pro-urokinase preparation will alter its thrombolytic properties. Therefore, not all pro-urokinase preparations can be assumed to be equivalent in efficacy or specificity.

Conclusions

On the basis of limited clinical data but extensive laboratory studies, some tentative conclusions may be ventured: 1) Pro-urokinase is a natural, plasminogen activator that is nonantigenic even at the pharmacologic dose and that has a fibrin specificity comparable with that of t-PA. 2) Pro-urokinase is rapidly inactivated by thrombin and its plasminogen activation is not potentiated by heparin; this may account for an apparent lower incidence of bleeding complications during thrombolysis compared with t-PA. 3) Pro-urokinase clot lysis in vitro has a lag phase that may explain an apparent lower efficacy when pro-urokinase is used alone. This lag phase can be attenuated by urokinase or t-PA and is followed by a more rapid second phase. Although a sigmoidal clot lysis curve has not been demonstrated in vivo, the long reperfusion times found clinically with pro-urokinase and the potentiation by urokinase are consistent with a similar phenomenon occurring. 4) Pro-urokinase appears to be complementary in its mode of action to t-PA, which seems to explain their synergy. 5) Thrombolytic efficacy, safety and economy are apt to be better served by the use of combinations of t-PA and pro-urokinase than by either activator alone. The specific combination suggested by the findings is that of a bolus injection of t-PA followed by an infusion of pro-urokinase.

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